DETECTIONOF BACTERIA

There is a rich diversity of microbial communities within any environmental system and many are not fully characterized. However, due to the diversity of microorganisms in the system, a variety of methodological approaches are required for a detailed analysis of community structure in a bid to unravel the complex antagonistic and synergistic effects between microbial communities in order to eventually improve process stability and efficiency of environmental monitoring. This can be achieved by the combined use of traditional culture-based, microscopic and molecular techniques.

Culture Techniques

To date, essentially all our knowledge of the microflora has been obtained from isolating organisms from an ecosystem by culturing and subsequently analysing them. This approach is still the mainstay for studies in many ecosystems. However, cultivation of microbes as a means to characterise microbial communities in a natural ecosystem has major shortcomings, as it is recognized that many microbes in different ecosystems cannot be cultivated by standard culture techniques. Despite the limitations, culture techniques are very powerful and absolutely essential to obtaining a complete picture of the diversity and role of the microbial ecosystem in different environmental zones. To study such a complex ecosystem, the combination of both culture and molecular based non-culture techniques are required. **culture-based techniques such as plate counts, membrane filtration and most probable number (MPN)** have an inherent limitation because only the viable population will grow to produce colonies under specific growth conditions whereas others that are important in the original sample do not proliferate. However, these traditional culturing methods employed with environmental samples also underestimate the total number of microorganisms due to the selective nature of the media used, the lack of detection of active but non cultivable (ABNC) microbes and failure to count microbes that are present as aggregates or associated with particles. Likewise, it is impossible to obtain pure cultures of most microorganisms in natural environment due to the complex syntrophic and symbiotic relationships that are abundant in nature. Contrarily, **direct microscopic methods e.g., DAPI epifluorescence microscopy** allows the direct observation and total enumeration of viable and non-viable microorganisms in the feedstock. Specifically, the identification and enumeration of methanogenic microbes can be achieved by epi-fluorescence microscopy, a technique based on their unique fluorescent pigment, factor F420. The coenzyme F420 shows autofluorescence (blue-green color) of methanogenic cells when excited by UV light. Hence, this autofluorescence serves as a diagnostic tool used to count autofluorescent methanogens. Results from this technique can be combined with those from **molecular methods** for a better overview of microbial population and structure in an environment.

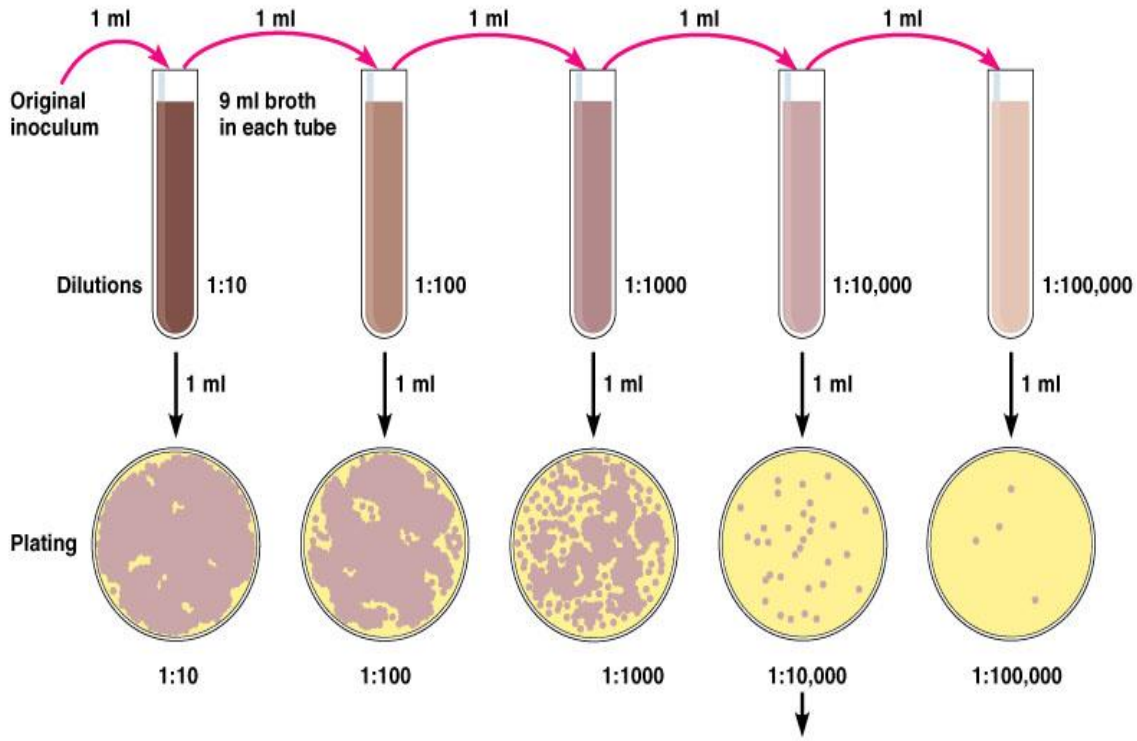
Plate count

The plate count method relies on bacteria growing a colony on a nutrient medium so that the colony becomes visible to the naked eye and the number of colonies on a plate can be counted. To be effective, the dilution of the original sample must be arranged so that on average between 30 and 300 colonies of the target bacteria are grown. Fewer than 30 colonies makes the interpretation statistically unsound whilst greater than 300 colonies often results in overlapping colonies and imprecision in the count. To ensure that an appropriate number of colonies will be generated several dilutions are normally cultured.

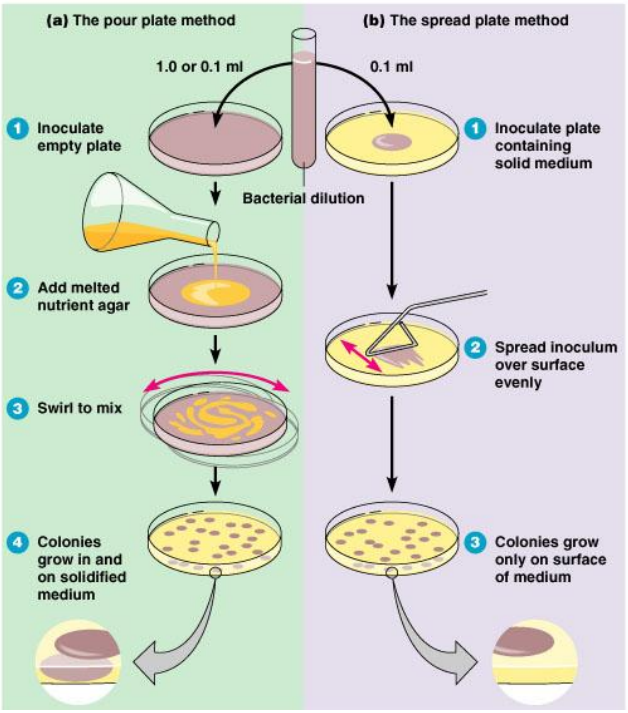
A standard plate count reflects the number of viable microbes and assumes that each bacterium grows into a single colony. Because it is impossible to say that each colony actually arose from an indivual cell (cells clump, fact of life) plate counts are reported as the number of colony-forming units (CFU) instead of the number of cells.

If the concentration of bacteria is too great the colonies will grow into each other and the plate will be uncountable.

To insure a countable plate a series of dilutions should be plated. The serial dilutions should give at least one countable plate in the series (25-250 or 30-300, depending on preference of the individual lab).



Calculation: Number of colonies on plate × reciprocal of dilution of sample = number of bacteria/ml
(For example, if 32 colonies are on a plate of $^{1}/_{10,000}$ dilution, then the count is 32 × 10,000 = 320,000/ml in sample.)



A plate count may be done on plates prepared by either the pour plate method or the spread plate method.

The total number of colonies is referred to as the Total Viable Count (TVC). The unit of measurement is cfu/ml (or colony forming units per millilitre) and relates to the original sample. Calculation of this is a multiple of the counted number of colonies multiplied by the dilution used.

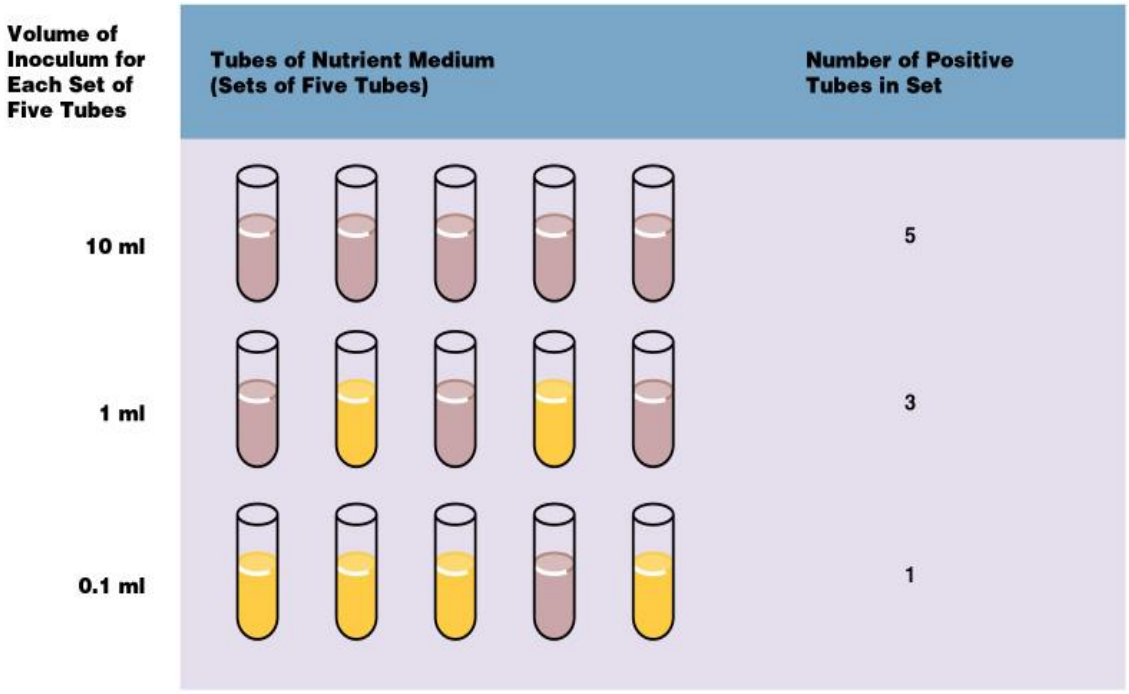
Membrane filtration

Most modern laboratories use a refinement of total plate count in which serial dilutions of the sample are vacuum filtered through purpose made membrane filters and these filters are themselves laid on nutrient medium within sealed plates. The methodology is otherwise similar to conventional total plate counts. Membranes have a printed millimetre grid printed on and can be reliably used to count the number of colonies under a binocular microscope.

MPN Method

The most probable number (MPN) method can be used for microbes that will grow in a liquid medium; it is a statistical estimation.

- A dilution series to no growth is prepared and the combination of positives is used to look the most probable number up in a table (see ***(b) MPN Table*** below).
- Used for microbes that won't grow on solid media or are grown in differential liquid media for identification purposes.



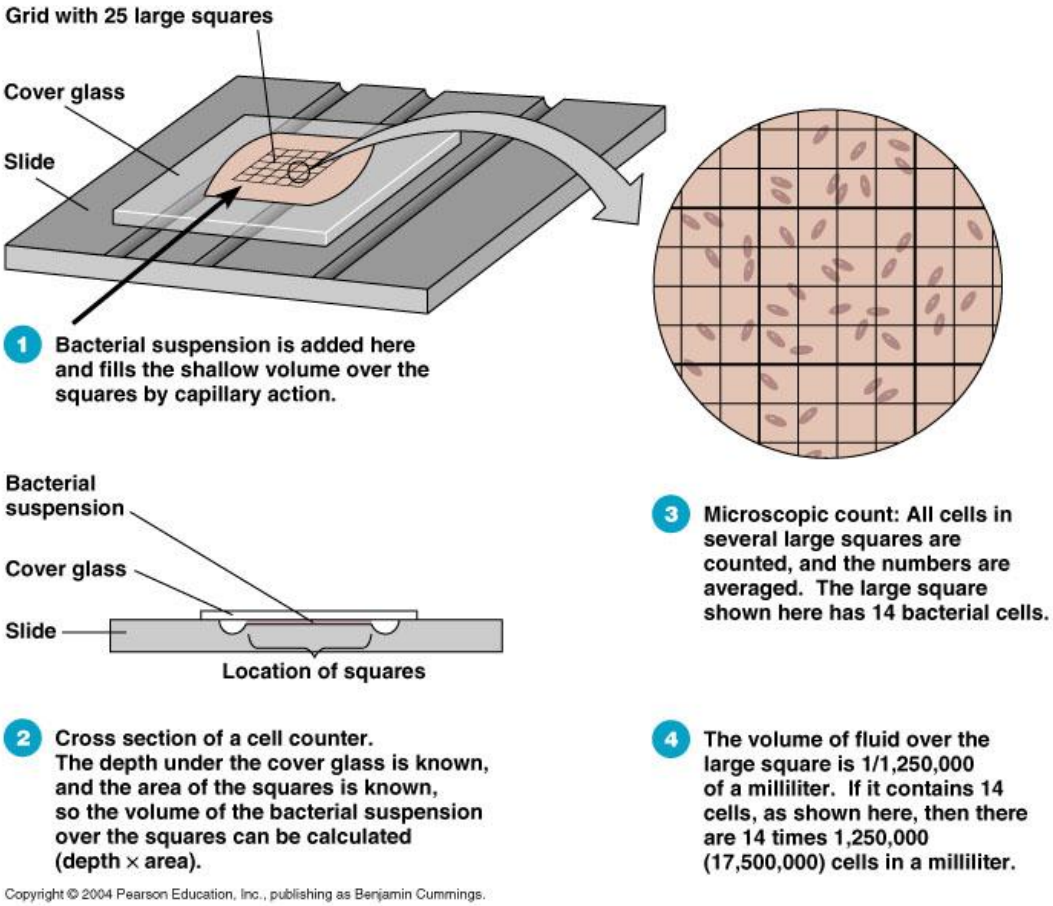
(a) Most probable number (MPN) dilution series

Combination of Positives	MPN Index/ 100 ml	95% Confidence Limits	
		Lower	Upper
4-2-0	22	9	56
4-2-1	26	12	65
4-3-0	27	12	67
4-3-1	33	15	77
4-4-0	34	16	80
5-0-0	23	9	86
5-0-1	30	10	110
5-0-2	40	20	140
5-1-0	30	10	120
5-1-1	50	20	150
5-1-2	60	30	180
5-2-0	50	20	170
5-2-1	70	30	210
5-2-2	90	40	250
5-3-0	80	30	250
5-3-1	110	40	300
5-3-2	140	60	360

(b) MPN table

Direct Microscopic Analysis

In a **direct microscopic count**, the microbes in a measured volume of a bacterial suspension are counted with the use of a specially designed slide.



Estimating bacteria by indirect methods

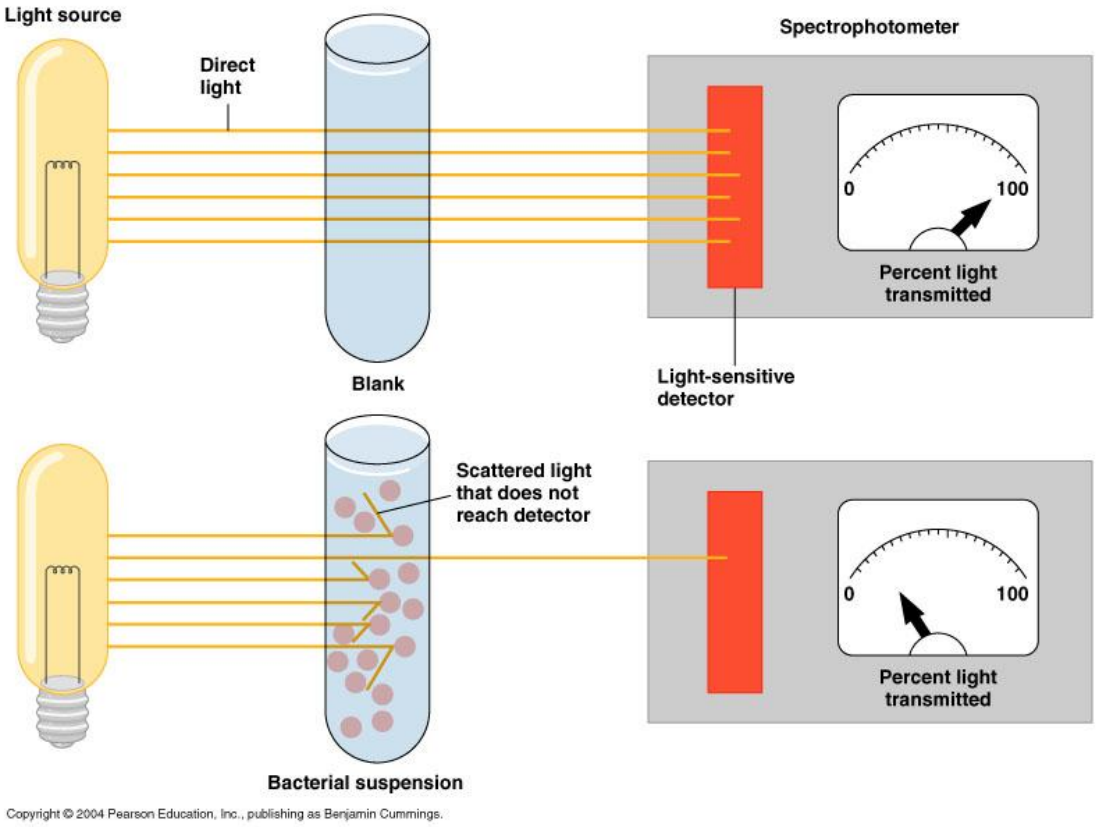
a) Spectrophotometer

A spectrophotometer is used to determine turbidity ("cloudiness") by measuring the amount of light that passed through a suspension of cells.

More cells = more turbidity; more turbidity = less light passing through the suspension

%T is percent transmission - fewer cells present (less turbidity) will allow more light to pass through, the %T is higher when the cell number is lower.

Absorbance is the opposite of %T. More light is absorbed when more cells are present - some people like this measure better because absorbance goes up as turbidity (or cell number) goes up.



b) Enzyme/Metabolite Analysis

Another indirect way of estimating bacterial numbers is measuring the metabolic activity of the population (for example, acid production or oxygen consumption). Measurement of specific enzymes and metabolites in faecal samples can indirectly give information on the presence of specific microflora, or to be more precise, on the metabolic activities of specific groups of microflora. This indirect approach can be quite rapid and therefore, can allow the analysis of a large number of individuals. It is also advantageous as it gives important functional information on the metabolic activities of the bacterial microflora.

Molecular Techniques for bacterial identification

Molecular techniques targeting particularly 16S rRNA genes (the only RNA component of 30S ribosomal subunit), can also be employed as conventional methods for identification of microbial community in a digester. These methods include cloning, fluorescent in situ hybridization (FISH), denaturing gradient gel electrophoresis (DGGE), single strand conformation polymorphism (SSCP), restriction fragment length polymorphism (RFLP), quantitative real-time PCR (qPCR) and DNA sequencing (Sanger and next generation sequencing methods).

Ex-situ Techniques

RFLP of the 16S rRNA Gene

This is a rapid technique, which involves amplifying the 16S rRNA gene using the PCR with primers targeted at universally conserved regions within this gene. The resulting amplicon is then restricted with an appropriate restriction enzyme and the resulting restriction fragments are size separated by agarose gel electrophoresis, forming a characteristic RFLP (part 'a' of figure). The choice of restriction enzyme depends on the particular genus and must be experimentally determined. As this is a PCR based technique, it can be carried out on very few cells, thus eliminating the need to culture colonies. This is a major **advantage** of all PCR based fingerprinting techniques. However, it has probably the highest reproducibility of all the PCR based fingerprinting techniques.

Pulse Field Gel Electrophoresis (PFGE)

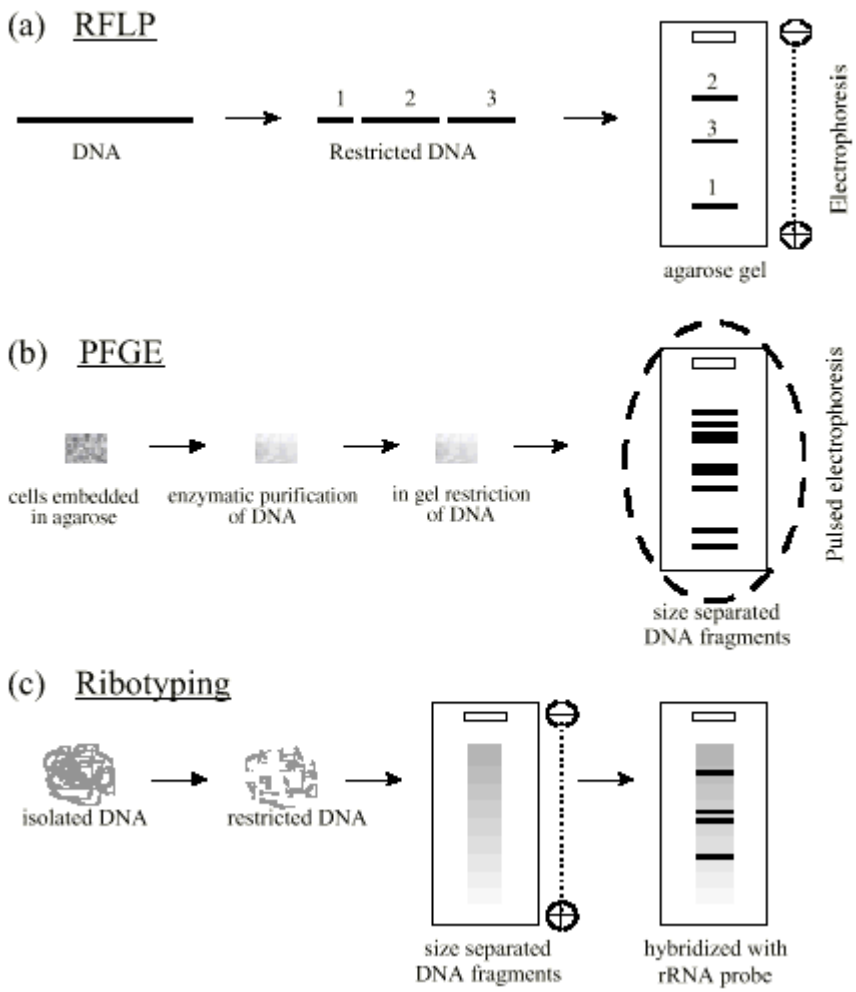
PFGE essentially means using an electrical pulse system to migrate very large fragments of DNA through an agarose gel. This technology can be applied to fingerprinting an isolate by digesting its genome into relatively few (5 - 50) large segments and separating them by PFGE. The fragments are obtained by digesting the genome with rare cutting restriction enzymes, which generally have an 8 bp recognition site or a 6 bp recognition site which may be statistically rare for the particular genome. Because the DNA fragments are large, they cannot be manipulated in aqueous solutions or they would be sheared mechanically. Therefore, all manipulations, including DNA isolation and restriction, are carried out on cells embedded in agarose plugs. The restricted fragments in the agarose plug are inserted then into a well in an agarose gel and separated by PFGE based on fragment size. The resulting pattern of DNA fragments is referred to as a restriction fragment length polymorphism (RFLP) and is highly characteristic of the particular organism. It should be noted that this fingerprint represents the complete genome and has the added advantage of detecting specific changes (DNA deletion, insertion or rearrangements) within a particular strain over time. This feature also makes this fingerprinting technique one of the most discriminatory (if not the most) techniques available. It is also very reproducible. **Disadvantages** of this technique are that colonies need to be cultured to obtain enough cells and that it is technically challenging, as well as labor intensive.

Ribotyping

A ribotype is essentially an RFLP consisting of the restriction fragments from a particular genome which contain rRNA genes. To obtain a ribotype for an organism, it must first be cultured to obtain enough cells for the procedure. Total DNA is then isolated and is totally restricted into multiple fragments, of sizes ranging from < 1 kb to > 20 kb, using a restriction enzyme with a frequently occurring recognition sequence, generally a 6 bp recognising enzyme. The restricted fragments are then separated by agarose gel electrophoresis and subsequently hybridised with a probe targeted to either the 16S, 23S or 5S rRNA genes (Figure-c part). In practice, probes to the 16S rRNA are the most commonly used. The hybridisation can be carried out directly in the gel using in gel hybridisation techniques, or alternatively on a nylon or nitrocellulose membrane following Southern transfer of the DNA from the gel to the filter. Following probe detection, restriction bands containing copies of the rRNA genes are visualised and the pattern of the band sizes represents a characteristic fingerprint. The basis of the technique is that bacteria generally contain multiple copies (up to

eight or more) of the rRNA genes throughout their genome, thus enabling the RFLP to be obtained. However, some bacteria contain as few as one copy of rRNA genes, thus limiting the effectiveness of ribotyping for fingerprinting these bacteria. Bacteria with a single copy of the rRNA operon are usually slow growing bacteria. An **advantage** of ribotyping is that a single rRNA probe can be used to type all bacteria. It is also very reproducible and effective. **Limitations** of this fingerprinting technique are, that it is not as discriminative as PFGE, requires culturing of bacteria and is labor intensive. However, the development of an automated ribotyping instrument (Dupont, Wilmington, DE) has increased the usefulness of this technique for the analysis of large numbers of isolates.

A related fingerpinting methodology is the use of a probe targeted to specific regions within a genome, such as a virulence gene or other unique characteristic of a particular organism. As this targets only a single genetic locus, its discriminatory power is low, but it is highly effective for analysing a population of organisms for specific traits. Probes can also be targeted at other sequences which may exist in multiple copies in a genome, such as insertion sequences (IS elements), thus enabling a characteristic RFLP to be generated.



In-situ Techniques

Fluorescence In Situ Hybridization

The detection of whole-bacterial cells via the labelling of specific nucleic acids with fluorescently labelled oligonucleotide probes is called fluorescence in situ hybridization (FISH). FISH requires no cultivation and cells can be fixed before analysis. Bacterial FISH probes are often primers for the 16s rRNA molecule. This molecule exists in multiple copies in the cell (up to 10E4-10E5) and is an excellent target for fluorescently labelled oligonucleotide probes which are directed against regions on the rRNA molecule specific for a bacterial group, genus or species. The small probes (16-20 nucleotides) will penetrate the bacterial cell wall and hybridise with the complementary target sequence. Evaluation of the test result is done by epifluorescence microscopy or flow cytometry. Compared to conventional cultivation techniques this method offers distinct advantages:

1. FISH allows the detection of one to three orders of magnitude more bacterial cells in samples. Even when using optimal media and growth conditions, generally less than 1-10% of the bacteria present in life samples will develop into detectable colonies.
2. FISH allows the study of the actual composition of the microbial community. When using cultural methods, over-representation as well as under-representation of populations can occur due to selectivity of the applied cultivation conditions.
3. FISH allows the in situ localisation and the study of spatial organisation of bacterial cells as they occur in their natural habitat. Unlike immunological methods for detection, FISH is not dependent on extracellular macromolecules that may only be expressed under certain cultivation conditions, but that are absent in other situations. FISH results are always definitive.
4. For FISH, cells need not be alive. Samples can easily be fixed and collected for later analysis. The intensity of the fluorescence is a direct measure for the activity of the cells. Inactive cells can be recognised by their low intensity fluorescence.

Procedure:

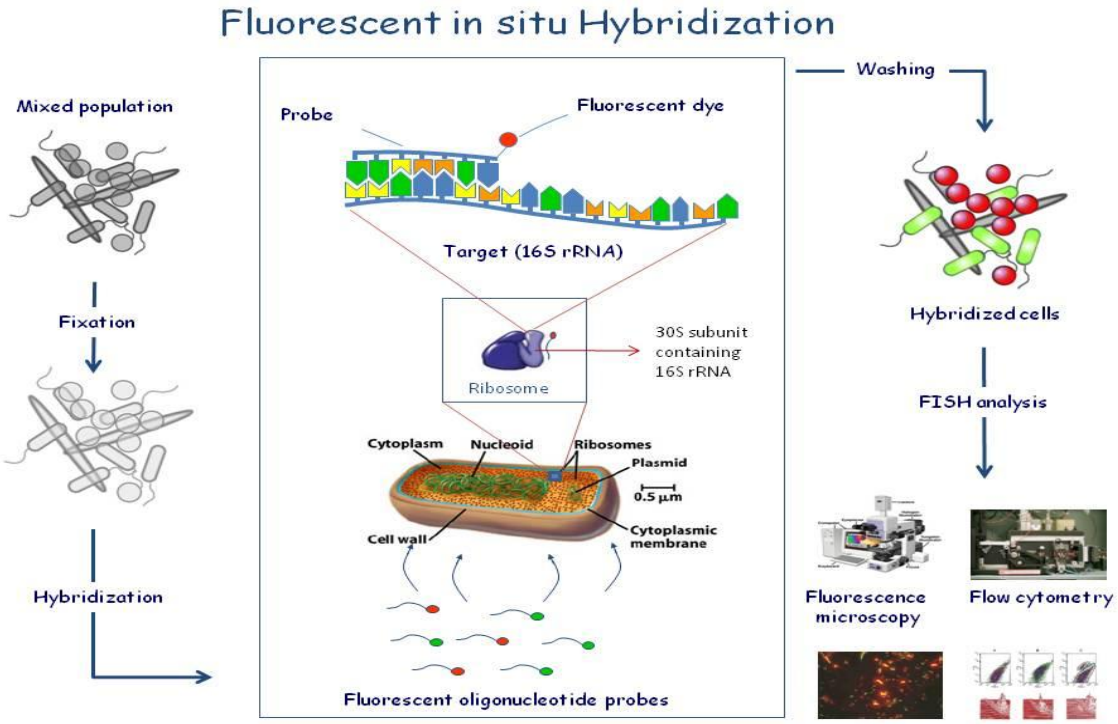
Fixation and Denaturation of Material

The DNA or RNA must be fixed onto a glass slide for stability. so as to allow the probe sufficient access to the targe maintain the structure as much as possible while giving the probe sufficient access to the target by permeabilizing the cells. While DNA is relatively stable and can be fixed easily, RNA is highly degradable, so extra care must be taken to fix the mRNA onto the slide as soon as possible after it is extracted. Possible fixatives include paraformaldehyde, formalin, or paraffin embedding. Cells must be treated with detergent or proteinases such as triton and RNAse-free proteinase K in order to permeabilize the membranes. The probe must have sufficient access into the cells so that it can bind with the target. The degree to which the cells are permeabilized affects the degree of specificity of ISH.

Probes

A variety of probes can be developed for ISH, depending upon the desired binding sensitivity. For DNA ISH, probes include double-stranded DNA, single-stranded DNA, and synthetic oligodeoxyribonucleotides. For RNA ISH, the probe can be single-stranded complementary RNA, called a riboprobe, that is synthesized by reverse cloning. Double-stranded DNA probes can be produced by PCR or replicated in bacteria. Double-stranded DNA probes must be denatured before hybridization and are often less sensitive because the single strands of DNA have a tendency to reanneal with each other. Single stranded DNA probes can be produced by PCR or reverse transcription of RNA. Oligonucleotide probes are

synthetically produced and are relatively small (20-40 basepairs). Oligonucleotide probes can fit easilty through permeabilized cell membranes and can be designed to be very specific. Complementary RNA probes can be produced from RNA. RNA-RNA interactions are very stable and resistent to RNAses, however before hybridization they are difficult to work with.
Once the probe is developed, it must be labeled so that the location of the target sequence can be detected. Probes can be labeled directly, where the reporter molecule is directly attached to the probe, or indirectly, where a specific antibody or labeled binding protein is used to detect another molecule that is attached to the probe sequence. Radioactively labeled probes were originally used for ISH and continue to be used because they can be synthesized and incorporated into the DNA or RNA easily and autoradiography is relatively sensitive. However, radioactively labeled probes have a limited shelf life and require additional safety procedures and autoradiography can take several days. Non-radioactively labeled probes (fluorescently labeled probes) are very popular, as the procedures are readily available and less time consuming. Typical radioactively-labeled probes use 32P or 35S isotopes. Fluorescin and Rhodamine can be used for direct fluorescently labeled probes, and biotin and digoxigenin can be used for indirect fluorescence labeling. Fluorescent labeling can allow two or more different probes to be visualized at the same time because of color differences.



Hybridization

The degree of specificity to which the probe hybridizes to the target sequence can be controlled by the design of the probe and the conditions of the buffer solution, including temperature, pH, and salt concentration. “High stringency” conditions will only allow hybridization of probes with very similar homology to the target sequence, while “low stringency” conditions will allow a probe to bind with less specificity. Hybridization mixtures usually have a small volume (about 10-20 ul total) with 50% formamide and hybridization typically occurs between 37-60 degrees Celsius (Polak *et al*., 1990).

Visualization

Several different techniques can be used to view where the probe has hybridized with the sequence of interest. Light field microscopy is most common and can be used for radioactively labeled probes or probes labeled with peroxidase or alkaline phosphatase. Fluorescent microscopes are used to view fluorescently labeled probes; the UV light excites the fluorescent die so that it can be detected through the microscope. Digital imaging systems are also used and can process the images and do quantitative measurements.

Molecular Advances for Typing and Phylogenetical Characterisation of the Intestinal Microflora

Classical culture techniques for the isolation of microbes from the human intestine is the sole source of intestinal microflora. Identification and characterisation of the resulting isolates by classical methods has many shortcomings, in particular, lack of accuracy and it is labour-intensive. The advent of molecular tools has greatly expanded the ability to reliably identify isolates and also to calculate the evolutionary relatedness between strains. Fingerprinting techniques (discussed in the next section), primarily DNA based, can be used for identification, but this strategy is limited by the extensiveness of the particular fingerprinting database. As databases for the different fingerprinting techniques grow, this approach will increase in usefulness. A major advantage of using a fingerprinting approach for typing purposes is its rapidity and, consequently it is conducive to analysing a large throughput of unknown isolates. A disadvantage, can be the sensitivity of the particular fingerprinting technique. While the sensitivity of the different fingerprinting techniques varies quite a bit, many common techniques do not have the sensitivity to differentiate between strains and in some cases, between closely related species. These low sensitivity fingerprinting techniques also have a limited ability to discern the phylogenetic relationship between isolates. These disadvantages limit the effectiveness of many fingerprinting techniques for accurate typing of unknown isolates and evaluating their phylogenetic relationships. It is however, an extremely powerful tool for monitoring known bacterial strains and is therefore the tool of choice for tracking the prevalence of certain intestinal isolates within a population.

16S rRNA Sequence Analysis

Accurate typing of unknown isolates is now achieved by sequence analysis of 16S ribosomal RNA (rRNA). This tool for classifying organisms and evaluating their evolutionary relatedness was first developed by Woese and coworkers (Woese, 1987). The available database of rRNA sequences is now extensive, which allows detailed studies to be made on the phylogenetic position of unknown isolates. This molecular phylogeny approach has revolutionized the field of microbial ecology and has allowed meaningful phylogenetic relationships between microbes in natural ecosystems to be discerned (Olsen *et al.,*1994). Technically, this is very feasible as the polymerase chain reaction (PCR) can be used to directly amplify the 16S rRNA gene directly from colonies using primers which are directed at universally conserved regions at both ends of the gene. The entire PCR amplicon, which is ~ 1.5 kb can then be directly sequenced and compared to the rRNA database (Figure 1).

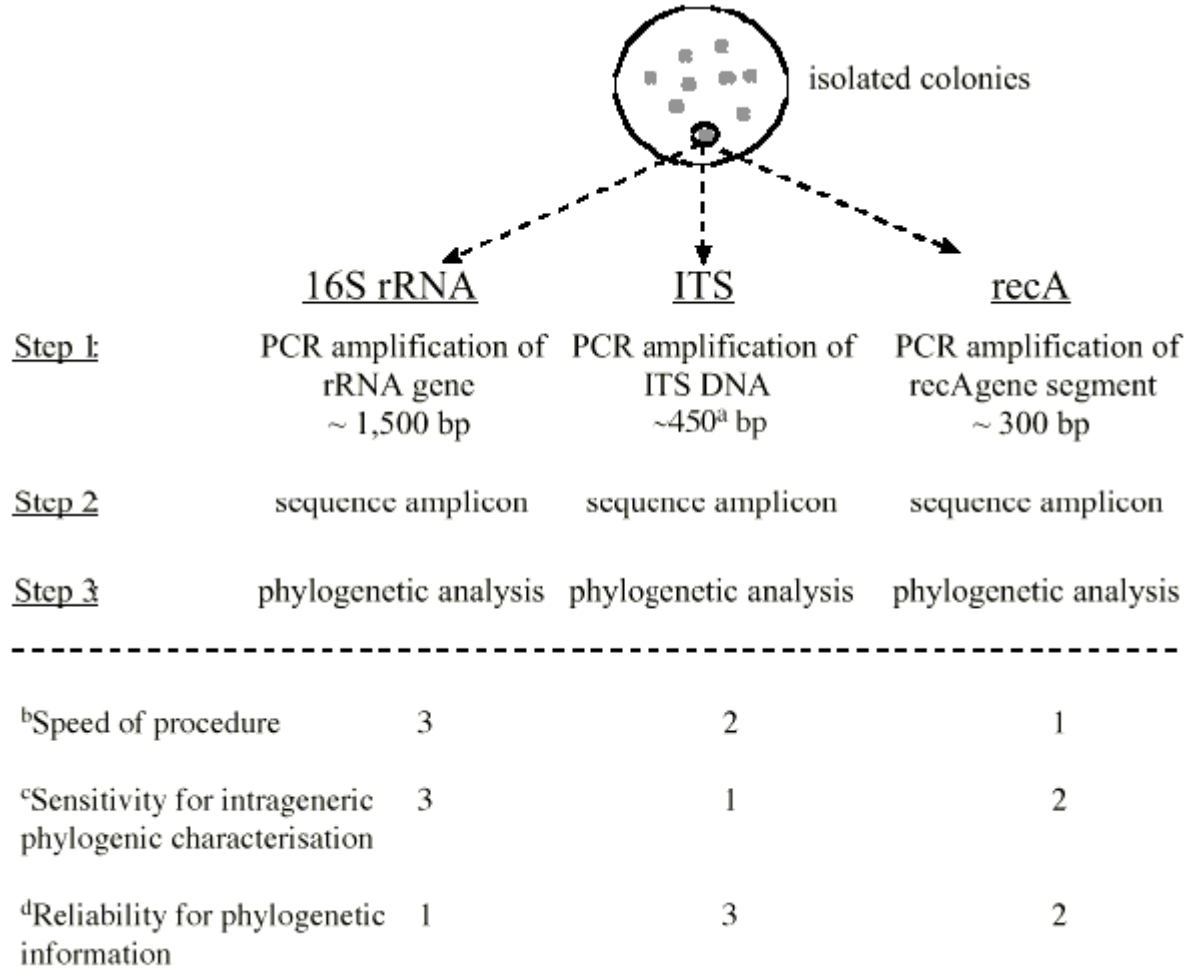


Figure 1. Comparison of the three sequence-based typing and phylogenetic characterisation approaches which have been used to characterise human intestinal isolates. The three sequences which have been used are 16S rRNA; sequence between the 16S and 23S rRNA genes, termed the internal transcribed spacer (ITS); and an internal portion of the *recA* gene. In each case the sequences were obtained via PCR using primers which are directed to universally conserved target sequences in each instance. [a], represents the average ITS size of 29 bifidobacteria isolates, calculated from Leblond-Bourget *et al*., (31); [b], numbers 1 - 3 represent rankings, with '1' representing the fastest procedure; [c], compares how sensitive the procedures are at differentiating isolates, with '1' representing the most sensitive; [d], represents theoretical and experimental evidence for the reliability of each molecule at evaluating phylogenetic relationships, with '1' indicating the most reliable. Note, the 16S rRNA molecule is the only one capable of evaluating phylogenetic relationships between multiple genera. Both the ITS and *recA* only have value for intrageneric characterisation.

This technology has greatly helped our understanding of the phylogenetic relationships between the major genera in the human intestine. The two major genera, *Bacteroides* and *Bifidobacterium*, are very heterogeneous and the use of 16S rRNA sequence analysis has contributed enormously to their phylogeny (Leblond-Bourget *et al.,* 1996; Shah and Collins, 1989). Understanding the phylogeny of bifidobacteria is particularly important, as members of this genus are prime candidates for inclusion in probiotic cultures for human consumption. Without comparative studies on the dominant bifidobacteria present in the human intestine, there is limited scientific rational for selecting specific strains for probiotic purposes.

ITS Sequence Analysis

Within the genus *Bifidobacterium*, the rRNA sequence is highly conserved (Leblond-Bourget *et al.,* 1996) and may not be sensitive enough for the desired level of comparative analysis that is likely to be needed for selection of worthwhile strains. Ideally, extensive phenotypic analysis would complement this approach and provide the level of analysis needed for rational strain selection. Indeed, this combined approach is thought to be the most powerful approach for understanding the true phylogeny of microbes and is emphasized in a recent review (Palleroni, 1997). However, this strategy is too labour intensive for high throughput of organisms from the intestinal ecosystem. To complement the rRNA sequence approach, analysis of another molecule, which is not as conserved as 16S RNA but still retains the characteristics of a meaningful phylogenetic marker, is required. Two important criteria for such a molecule are, that it is universally present in bacteria and it has high sequence conservation, which illustrates that sequence changes are less influenced by temporary environmental changes. The region between the 16S and 23S rRNA genes, termed the internal transcribed spacer (ITS), has been used for a more detailed analysis of bifidobacteria (Leblond-Bourget *et al.,* 1996). This molecule is universally present in bacteria, but can exhibit very low sequence conservation (Barry *et al.,* 1991), thus limiting its accuracy as a phylogenetic marker. In addition the ITS regions within the same bacterial strain can exhibit heterogeneity (Christensen *et al.,* 2000; Garcia-Martinez *et al.,* 1996). However, the molecule is technically very feasible to obtain as PCR can be used to amplify the molecule directly from colonies using primers directed at universally conserved regions within the bordering 16S and 23S rRNA genes (Figure 1). Leblond-Bourget *et al*., (1996) did evaluate the sequence analysis of this molecule for further characterising bifidobacteria and did find it gave much more sensitivity than the rRNA analysis. More recently, Tannock *et al.* (1999) demonstrated its usefullness for identification of intestinal *Lactobacillus* spp.

***recA* Gene Sequence Analysis**

Recently, a short segment of the *recA* gene has emerged as a potential candidate for a sensitive molecule for determining intrageneric phylogenetic relationships and is amenable to large scale analysis of a natural ecosystem, such as the human intestine. It possesses the important criteria of being universally present in bacteria and also being highly conserved. The *recA*gene encodes the RecA protein, which plays vital roles in recombination, DNA repair and the SOS response (Roca and Cox, 1997). Studies have established that meaningful bacterial phylogenetic relationships can be obtained by sequence analysis of the RecA protein (Eisen, 1995; Karlin *et al.,* 1995). These studies highlighted the possibility that a segment of the *recA* gene might be a useful molecule for phylogenetic analysis within a particular genus. This concept was applied to the genus*Bifidobacterium* in a recent study by Kullen *et al*., (1997a). The molecule was obtained from both type and intestinal bifidobacterial isolates direct from colony isolates using PCR with primers directed to regions within the *recA* gene, which are universally conserved in bacteria. This approach yielded a fragment of ~ 300 kb, which could be rapidly sequenced using a single sequence reaction from either end (Figure 1). The phylogenic relationship obtained by sequence analysis of this short segment of the *recA* gene, compared favorably with the analysis from the complete rRNA gene. Given the rapidity of obtaining the sequence information of this *recA* molecule, coupled with its theoretical and experimentally substantiated role as a meaningful phylogenetic molecule, it is potentially a very valuable tool for comparative phylogenetic analysis of human intestinal bifidobacterial isolates.

Modern Approaches for Monitoring the Distribution and Prevalence of Specific Microbes in the Intestine

Rapid analysis of colony isolates is an important feature of any approach to study the intestinal microflora on a large scale by culturing methodology. Fortunately, a number of rapid detection or fingerprinting approaches have been developed for this purpose, which can provide valuable information on the range of

different types of isolates. Many of these rapid techniques also provide information on how related different strains are to one another. While this comparative analysis is generally less sensitive than the sequence based methods discussed in the previous section, it is a valuable first step to divide the isolates into broader groups, prior to the more elaborate sequence based methods.

Phenotypic Fingerprint Analysis

Fingerprint techniques have been developed which are directed at both phenotypic and genotypic characteristics. Although phenotypic fingerprints can be obtained, these are usually less sensitive and changes in the fingerprint may not necessarily mean a different organism, but rather could be due to a change in expression of the particular phenotypic trait. Examples of phenotypic fingerprints are polyacrylamide gel electrophoresis of soluble proteins, fatty acid analysis, bacteriophage typing and serotyping. The most rapid and useful of these procedures is serotyping, as colonies can be directly typed, without sub-culturing, by colony hybridization with a monoclonal antibody specific for a particular genus, species or strain. This strategy has been applied for the analysis of two *Bacteroides* species in different human intestines (Corthier *et al.,* 1996).

Genotypic Fingerprint Analysis

The recent development of multiple genotypic fingerprinting methodologies has been a major advantage for deciphering the complex human intestinal ecosystem. While all possess limitations, each can contribute to our understanding of the diversity of the different types of dominant microbes present in the intestine of different individuals. The first molecular detection system developed was hybridization with a nucleic acid probe targeted at a specific DNA sequence. While this is an elementary fingerprinting technique, more sophisticated methodologies have since been developed. Those which are potentially useful for the study of the human intestinal microflora are discussed below and a comparison of these fingerprinting techniques is summarized in Table 1.

Colony Hybridization with Nucleic Acid Probes

A nucleic acid probe is a labelled single-stranded nucleic acid that can specifically hybridize (bind) to its complementary sequence. Probes therefore can target specific sequences in a genome. These target sequences are generally chosen such that they are unique to the particular genus, species or strain. Technically, the procedure is rapid as colonies can be directly probed, by lysing the colony to expose the nucleic acid content and allowing access for the probe. The label on the probe can be either radioactive, enzymatic or fluorescent, which can be readily detected. The selection of probes however, is the key to success with this approach, as any cross reactivity can give ambiguous results. Probes can be obtained using either a shotgun or a directed approach. The shotgun approach is to randomly isolate DNA fragments and test them for probe reactivity against a bank of isolated strains. This approach has been used to obtain strain- and species-specific probes for bifidobacteria (Ito *et al.,*1992; Mangin *et al.,* 1995) and a species specific-probe for *Bacteroides vulgatus* (Kuritza and Salyers, 1985). However, these probes have yet to be tested in large scale studies of human intestinal isolates.

The directed approach to probe selection leaves less to chance, as it relies on choosing probes directed at target sequences which are thought to be unique to the particular microbe or group of microbes under study. One strategy is to identify enzymes unique to a group of organisms and direct probes at targets within the enzyme gene sequence. A potential example would be the bifidobacterial enzyme, fructose-6-phosphate phosphoketolase (F6PPK), which is used by members of this genus to metabolise carbohydrates via a unique pathway often called the fructose-6-phosphate shunt. As this enzyme is unique to this genus (with the possible exception of *Gardnerella*), it potentially is a good source of probes for this

genus and should afford an effective means for tracking these bacteria in the gastrointestinal tract. However, the sequence of this gene is not yet available.

Generating short (~ 20 bases) oligonucleotide probes directed at regions of the rRNA is the most common means of obtaining genus- and species-specific probes. This approach is possible as there are some short variable sequence regions within this molecule, which can distinguish to the genus- or species-level. During the probe design process, probes can be tested against the extensive database of rRNA sequences using computer models. With the correct design procedure, the resulting probes should have a low cross reactivity. Using this strategy, genus specific probes have been designed and evaluated for detection of*Bacteroides* (Dore *et al.,* 1998), *Bifidobacterium* (Kaulmann *et al.,* 1997), *Lactobacillus* (Sghir *et al.,* 2000) and *Clostridium* (Sghir*et al.,* 2000) from human faecal samples. Species specific probes for bifidobacteria (Yamamoto *et al.,* 1992), *Bacteroides*(Kreader, 1995) and *Lactobacillus* (reviewed, Schleifer *et al.,* 1995) have also been developed.

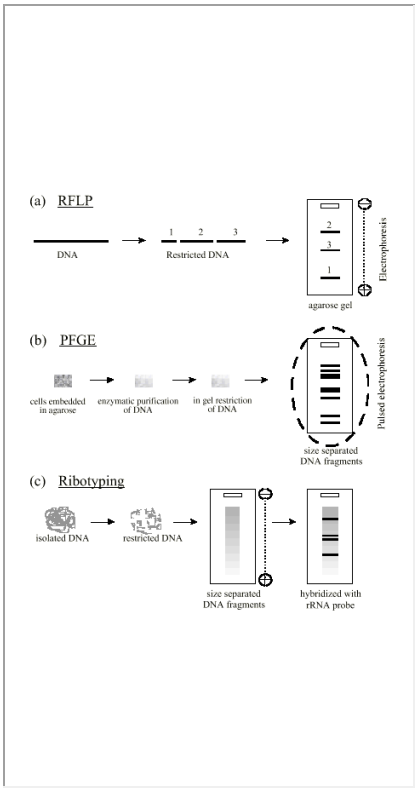


Figure 2. Illustration of commonly used fingerprinting techniques. (a) Demonstration of restriction fragment length polymorphism (RFLP). The DNA fragment is restricted into 3 pieces (1, 2 and 3) by a restriction enzyme. The restricted DNA is then inserted into a well in an agarose gel and the fragments are separated by electrophoresis. The separation is based on fragment size, with the smallest fragments migrating further into the gel. This pattern, termed RFLP, represents a characteristic fingerprint of this DNA fragment. (b) Pulse field gel electrophoresis (PFGE) to separate large restriction fragments (50 - 1,000 kb) into a characteristic RFLP. Whole cells are embedded in agarose and DNA is enzymatically purified and restricted by rare cutting enzymes *in situ*. The agarose plug is then inserted into a well in an agarose gel and the restricted fragments are separated by an electric current which pulses from different angles. (c) Demonstration of ribotyping. DNA is isolated from a cultured isolate, restricted and size separated in an agarose gel. The gel is then hybridized with labeled rRNA probe, which binds to fragments containing copies of the rRNA operon. Following probe detection, fragments with bound probe are visualised, forming a characteristic RFLP.

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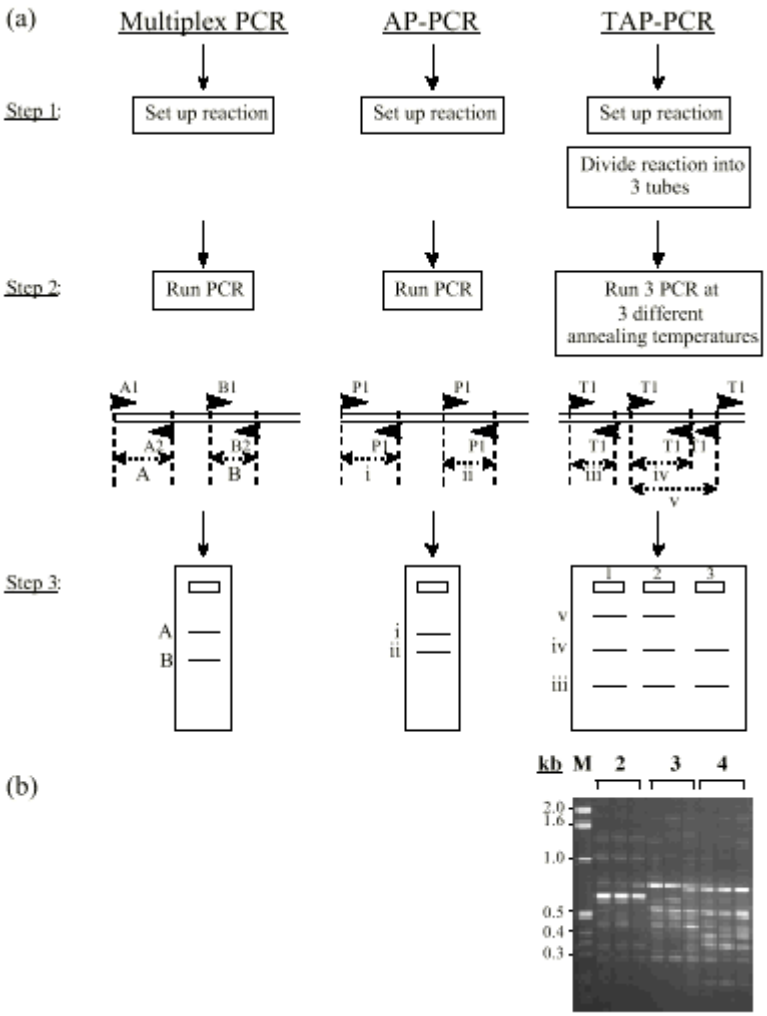
Multiplex-PCR

The PCR, which was developed by 1993 Nobel prize recipient Kary Mullis (reviewed, Mullis, 1990), is one of the most useful molecular tools of modern time. In its simplest form, PCR is used to amplify a specific DNA sequence over a billion-fold from a single copy, using a thermostable DNA polymerase (usually *Taq* DNA polymerase), deoxynucleotides (dNTP) and two primers, whose sequence is complementary to either end of the targeted sequence. This is achieved using multiple cycles of the PCR, generally 30 - 40. During each cycle of the PCR, the reaction tube is first heated to ~ 94 °C, which denatures the double stranded template DNA. The temperature is then dropped to < 55°C (typically), which

allows the primers to anneal to their target sequences, and then to 72 °C to enable the *Taq* polymerase to extend from both primers, thus creating a duplicate copy of the DNA region between the two primers. This duplicated region is generally < 5 kb, although it is now possible to amplify much larger fragments. As each duplicated copy becomes template for the next cycle of PCR, the amplification is exponential, where by a single copy is potentially amplified to 2^n (n = number of cycles). Therefore, in a typical PCR of 35 cycles, ~ 3.4×10^{10}copies can potentially be generated.

In a multiplex PCR, more than one set of primers is included to enable the simultaneous amplification of a number of target DNA regions (Figure 3a). The more target regions amplified, the more reliable the technique. A disadvantage of the technique is that prior sequence knowledge is required and it is technically challenging to design optimal reaction conditions. It was recently adapted for the reliable identification of human *Lactobacillus* species (Song *et al.,* 2000).



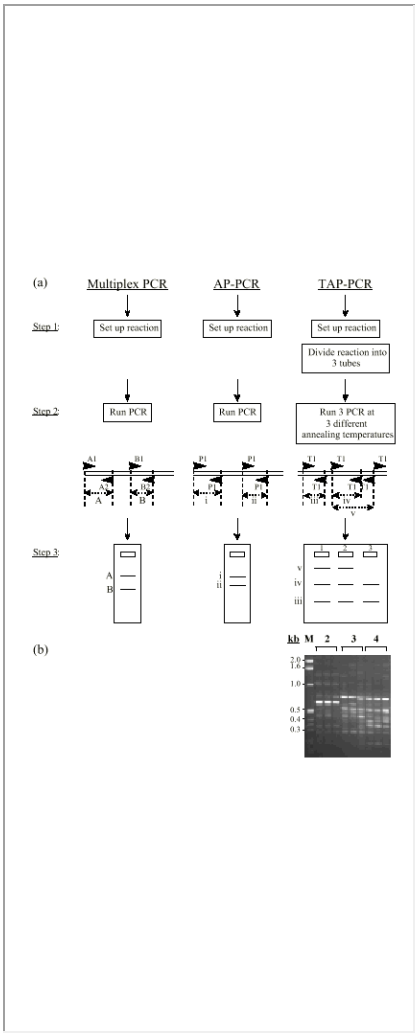


Figure 3. (a) Illustration of three PCR fingerprinting techniques. Step 1: The reaction in each case contains template, primers, dNTP and *Taq*polymerase in an appropriate buffer. The template can be a portion of a colony, which has been disrupted by microwave, physical shearing with glass beads, or other cell disruptive process. The primary difference between the three reactions is the primer(s). The multiplex PCR procedure contains primers A1 and A2, which are specifically targeted at sequences bordering DNA fragment A and primers B1 and B2, which target fragment B. The AP-PCR contains a 10 base primer (P1), whose sequence is arbitrarily chosen. TAP-PCR contains an 18 base primer (T1), whose sequence is based on a conserved region of the 16S rRNA. Step2: The annealing temperatures for both the multiplex and AP-PCR need to be experimentally optimized. The annealing temperatures for the TAP-PCR are 38, 40 and 42°C respectively. DNA regions indicated by dotted lines can be amplified. Step 3: Agarose gel separation of amplified fragments to form characteristic banding patterns. A and B indicate the fragments targeted by the multiplex PCR. Roman numerals indicate the arbitrary primed products. Lanes 1 - 3 indicate the 3 reactions of TAP-PCR. Bands which appear in at least 2 out of 3 lanes are considered reproducible. (b) Example of TAP-PCR fingerprint of*Bifidiobacterium breve* ATCC 15701 (lane 2), *B. infantis* ATCC 15697 (lane 3) and *B. infantis* ATCC 15702 (lane 4). The annealing temperatures for each TAP-PCR of each strain were 38, 40 and 42°C respectively. M, 1 kb ladder (BRL).

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Arbitrary Primed (AP) PCR

AP-PCR differs from conventional PCR in that only a single short primer (usually 10 - 12 bases), whose sequence is arbitrarily chosen, is used. To enable the primer to anneal to the template DNA, the stringency of the reaction is reduced, allowing the primer to bind to regions where it exhibits nearest homology. When these primer binding sites are within a few thousand bases and are on opposite strands, the DNA region in between can be amplified (Figure 3a). The more products which are amplified, the more discriminatory the technique. This fingerprinting technique was first described in 1990 and was termed AP-PCR (Welsh and McClelland, 1990) or RAPD (Williams *et al.,* 1990). As this rapid technique is very discriminative and can be applied to organisms for which no sequence information is known, numerous protocols have been developed for many bacterial genera. The major disadvantage of the procedure is that subtle changes in reaction conditions can change a banding pattern, thus compromising the reproducibility of the technique.

Triplet Arbitrary Primed (TAP) PCR

The low reproducibility of arbitrary priming results from unintended changes in reaction conditions. By purposely introducing specific changes to the reactions in three otherwise identical reactions, the amplicons which are susceptible to changes in the reactions can be identified. This is the basis of TAP-PCR (Cusick

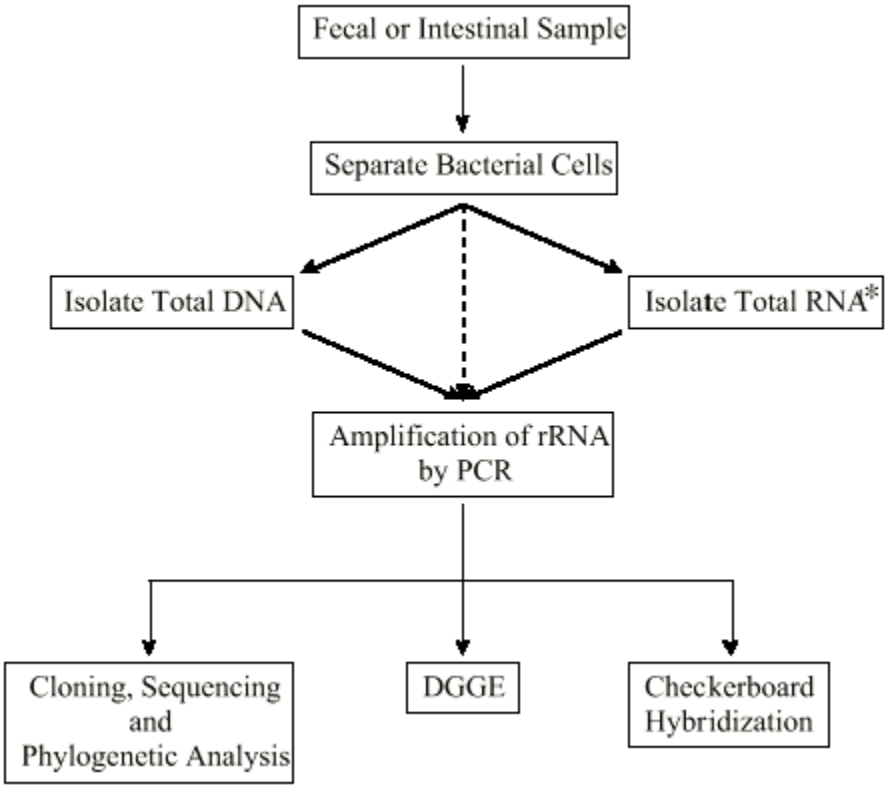
and O'Sullivan, 2000). The triplet reaction is conducted in parallel at three different annealing temperatures (38, 40 and 42°C) and following gel electrophoresis of each reaction, the banding patterns are compared. Bands which are present in at least two lanes are considered resilient to small changes in reaction conditions and are therefore considered in the fingerprint analysis (Figure 3a). The technique can be discriminative to the species and strain level (Figure 3b). It maintains the significant advantages of AP-PCR, but increases the confidence limits of the fingerprinting result (Table 1).

Culture-Independent Molecular Approaches to Analysing Intestinal Microflora

The advent of culture-independent molecular techniques has in many ways revolutionised the field of microbial ecology. However, to date these tools have not been used to a large extent for the analysis of the human intestinal microflora. A probable reason for the lack of impact these tools have had in the study of this ecosystem, is that unlike many other natural environments, culture techniques have been more successful at identifying a large portion of the intestinal microflora. Recently, these molecular techniques have begun to be directed to the human intestine and should unveil many more mysterys of its complex microbial microflora. The techniques developed to date rely on directly amplifying 16S rRNA from faecal samples using PCR. Generally, faecal samples are first enriched for bacterial cells by differential centrifugation and can be used directly in PCR, or total DNA or RNA can first be extracted. The procedure can target the rRNA genes by using DNA, or cells, in a standard PCR. As rRNA is often thousands of times more plentiful then rRNA genes, total RNA can also be used as template if the enzyme reverse transcriptase is also included. This enzyme can generate a complementary copy of the single stranded rRNA during the first cycle, thus creating a double-stranded template for the PCR. Targeting rRNA, rather than DNA, can be used to preferentially identify bacteria which are metabolically more dynamic, as faster growing bacteria have greater amounts of rRNA.

There are limitations with these rRNA based culture-independent techniques, regarding their estimations of biodiversity in natural habitats. One limitation concerns the disparity in the number of rRNA operons in different bacteria. Clearly, an organism with one copy of rRNA genes will be under represented compared to organisms with eight or more copies. The disparity is magnified if rRNA is used as the template for the PCR. Another limitation concerns the use of universal primers for the amplification of the rRNA product. These primers are not identically homologous to all bacteria and will not amplify all rRNA products with the same efficiency. This can result in a disparity in the biodiversity in favour of those organisms more conducive to PCR with the primers used. To help control for this limitation, different sets of primers targeting different universally conserved regions within the rRNA can be used.

Following isolation of the amplified 16S rRNA product, there are three strategies that are presently being used for the analysis of the human intestinal microflora (Figure 4): cloning and sequencing of individual rRNA genes; separation of individual rRNA products by denaturing gradient gel electrophoresis (DGGE); and checkerboard hybridization with specific probes.



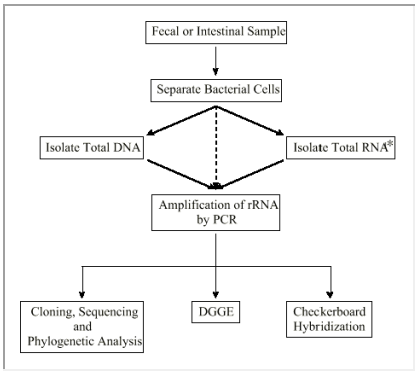


Figure 4. Outline of molecular approaches for culture-independent analysis of the human intestinal microflora.

*** to use total RNA as a template for PCR, a reverse copy of the rRNA gene needs to be generated using the enzyme reverse transcriptase. This process is termed RT-PCR.**

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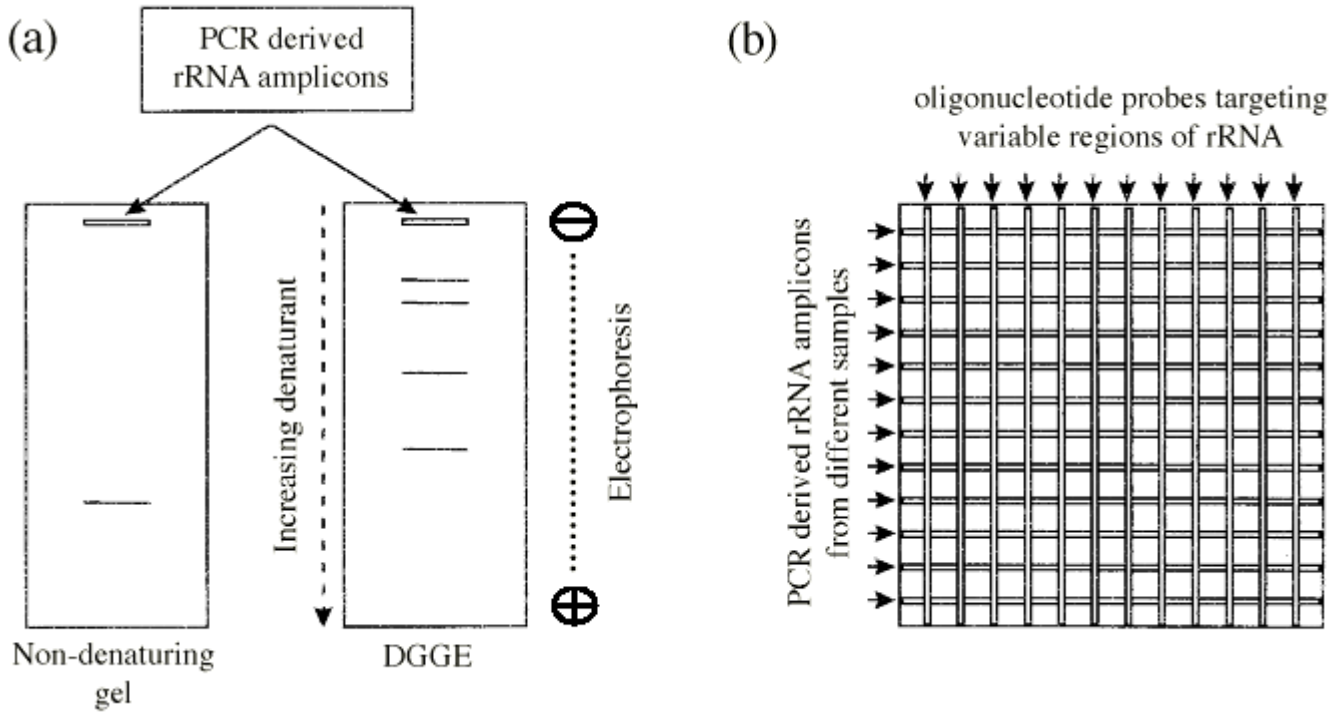
Sequencing of Individual rRNA Genes

The amplification of a 16S rRNA product from a faecal sample results in a heterogenous mix of products, within the amplicon. Cloning the amplicon into a standard sequencing vector can result in a bank of individual rRNA gene clones. These clones can then be sequenced and phylogenetically analysed. This strategy has been fundamental to the discovery of numerous new organisms, as well as to our present taxonomic division of all organisms into *Eucarya*, *Archaea* and *Eubacteria*. Recently it has been applied to the analysis of human intestinal microflora and preliminary indications are that many of the sequences identified were from novel organisms (de Vos *et al.,* 1997; Suau *et al.,* 1999; Wilson and Blitchington, 1996). This is extremely noteworthy, as it suggests that culture techniques may not be as effective at analysing the biodiversity of this environment as was generally perceived. The further use of this molecular tool to study the diversity of organisms in the human intestine, will have a major impact on the field of

probiotics, as knowledge of the balance of the microflora in different individuals is paramount to understanding their functional role in intestinal health.

Denaturing Gradient Gel Electrophoresis (DGGE)

This procedure can separate individual rRNA genes from the universally amplified product. Although all the individual rRNA species within the amplicon are of the same length, electrophoresis through a linearly increasing gradient of denaturants can separate the products of different sequence (Fischer and Lerman, 1979). The principle is based on the melting of rRNA genes at specific denaturing points based on their sequence. Therefore, each individual sequence will begin to melt at a characteristic denaturing point. The melting changes the conformation of the DNA molecule, slowing its migration through the gel (Figure 5a). Urea and formamide are generally used to form the denaturing gradient. However, temperature can also be used, thus creating a temperature gradient gel electrophoresis (TGGE). When an individual rRNA gene begins to melt, its migration slows and it becomes separated from the PCR amplicon. Further migration of the gene through the denaturing gradient, however, could result in the double-stranded DNA becoming denatured into ssDNA products. To prevent this occurring a GC clamp, consisting of 30 - 50 "G" and "C" bases attached to the 5' end of one of the primers used to amplify the rRNA product, can be used. As G/C rich DNA regions are resilient to melting, this tag can maintain the integrity of the double stranded rRNA genes.



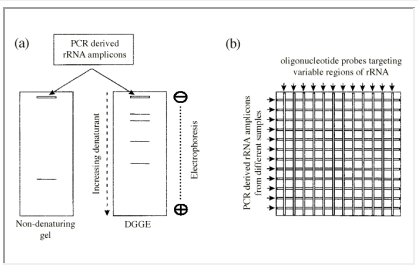


Figure 5. (a) Illustration of density gradient gel electrophoresis (DGGE) of a PCR derived rRNA amplicon from four different bacteria. Gel electrophoresis in a non-denaturing gel results in a single band, whereas electrophoresis through a gel containing increasing concentrations of a denaturant results in the separation of the four different products, based on their sequence

	dependent melting patterns. Note, to maintain the double stranded integrity of the products, a G/C clamp is generally included in one of the primers used in the amplification. (b) Illustration of the checkerboard hybridization format for analyzing large numbers of samples with multiple probes. Each vertical channel contains a different bound probe and each horizontal channel contains a PCR derived rRNA amplicon from a different individual. In this example, 144 different hybridizations can be carried on one membrane.

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This approach can potentially provide a fingerprint of the complexity of the intestinal microflora of an individual. The use of this technique will enable rapid detection in the makeup of an individuals flora over time (Zoetendal *et al.,* 1998). It can also reveal the presence of new isolates, such as ingested probiotic strains that may be present (de Vos *et al.,* 1997).

Checkerboard Hybridization

The ability to rapidly detect certain microbes among an individual's intestinal microflora is very useful, particularly when investigating the distribution of certain species among a large population of individuals. One approach which has been used to detect a probiotic *Bifidobacterium* strain in faeces, was to use species-specific primers directed to the 16S rRNA gene and amplify directly from faecal samples (Kok *et al.,* 1996). An alternative and potentially more sensitive approach would be to use universal rRNA primers to amplify the rRNA amplicon from faeces and subsequently probe the amplicon with species-specific oligonucleotide probes. This approach can be adapted to the analysis of multiple samples with multiple probes at once using checkerboard hybridisation (Socransky *et al.,* 1994). This procedure, which is illustrated in Figure 5b, can potentially enable large numbers of individuals to be screened for specific microflora in a short time. The effectiveness of the technique, however, depends on the specificity of the probes used. The technique also depends on the probes having similar melting temperatures, as all must undergo the same hybridization stringency. At present there are very few probes available with the desired level of specificity and melting stringency. However, future development of more strain-specific probes should render this approach very useful for the analysis of specific microflora in multiple individuals.

***In situ* Analysis of Intestinal Microflora**

The ability to be able to obtain information on single cells *in situ* in faecal or intestinal samples is very intriguing. This event is now feasible, primarily due to the development of sensitive fluorescent labels, which enable probes to be visualised by fluorescent microscopy. Visualisation of specific strains at the single cell level *in situ*, can be achieved by prokaryotic *in situ* PCR (PI-PCR) or fluorescent *in situ* hybridization (FISH).

PI-PCR

In situ PCR relies on the amplification of specific gene sequences inside intact cells with primers that have fluorescent tags (reviewed, Long and Komminoth, 1997). This technique has mostly been applied to eukaryotic cells, but has recently been adapted for bacteria. Hodson *et al*., (1995) developed the PI-PCR to enable *in situ* visualization of individual bacterial cells in natural environments. In this study, primers were developed based on specific genes within the bacteria of interest, and these fluorescent tagged primers were used in a PCR on a glass slide containing a preparation of the cells. Following amplification, individual

cells containing the targeted gene could be visualized by fluorescent microscopy. Recently, Tani *et al*., (1998) used an improved fluorescent label and demonstrated the efficacy of the procedure for visualising specific bacteria at the single cell level in a natural environment. This technique has yet to be applied to the human intestinal microflora. However, it offers tremendous promise, particularly as it can potentially be adapted to determine what individual cells are expressing specific genes.

FISH

An alternative to *in situ* PCR amplification is to hybridise fluorescent labelled oligonucleotide probes directly to cells fixed on a glass slide (reviewed, Amann, 1995). The fixing process permeates the cells to allow the short probes to access the nucleic acid inside the cell. This hybridization can be carried out on glass slides and the cells with the hybridised fluorescent probe can subsequently be visualized by fluorescent microscopy. Using non-specific probes to the 16S rRNA, FISH has indicated the number of bacteria in human faecal samples is approximately ten-fold higher than estimated using standard culture techniques (Harmsen *et al.,* 2000; Langendijk *et al*., 1995). The technique has also been evaluated for the detection of specific mRNA species within cells (reviewed, de Vos *et al.,* 1997). This technology, in conjunction with PI-PCR, can potentially reveal what specific genes are expressed by the microflora *in situ* in the human intestine.

Conclusion

This decade has seen the emergence of numerous molecular approaches for the analysis of different aspects of the human intestinal microflora. Their use, in conjunction with traditional culture methods, has already significantly enhanced our knowledge of this ecosystem. The recent adaptation of culture-independent molecular tools to the human intestinal microflora, offers further potential for revealing a more detailed picture of the true complexity of this unique environment. These techniques also have the potential to explore the functionality of certain microbial traits in the intestine, particularly as *in situ* mRNA detection systems become more sophisticated. The next few years should see the impact of these approaches on our understanding of this ecological niche. The contribution of these studies to the field of probiotics is enormous, as stringent scientific studies are the key to providing the necessary scientific substantiation for the efficacy of potential probiotic bacteria on intestinal health.

Figure 3

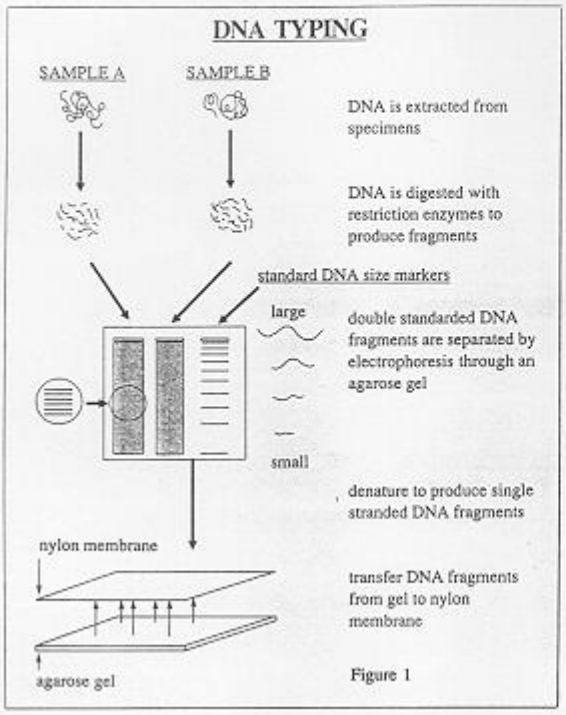

Figure 1

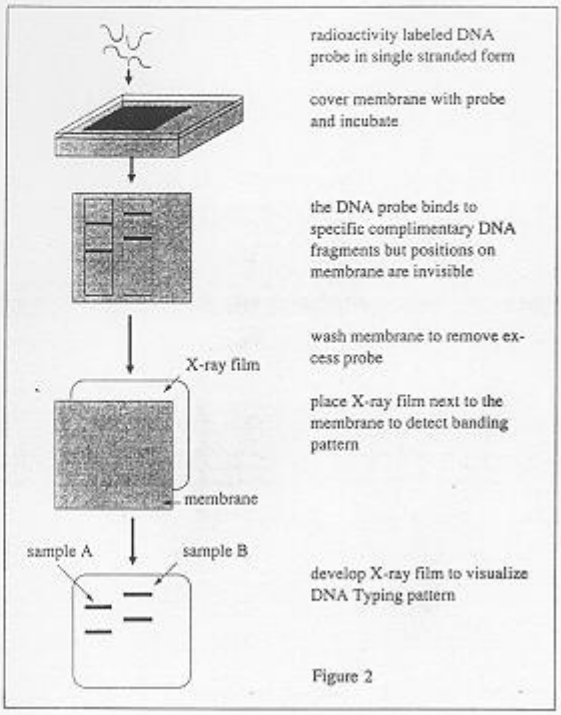

Figure 2